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## **Adaptation of opossum kidney cells to luminal phosphate: effects of phosphonoformic acid and kinase inhibitors**

Thomas, Linto ; Wagner, Carsten A ; Biber, Jürg ; Hernando, Nati

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Original Paper

# Adaptation of Opossum Kidney Cells to Luminal Phosphate: Effects of Phosphonoformic Acid and Kinase Inhibitors

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## Key Words

Proximal tubule • Reabsorption of phosphate • Phosphate sensing • OK cells

## Abstract

**Background/Aims:** Renal reabsorption of inorganic phosphate (Pi) is mediated by SLC34 and SLC20 Na<sup>+</sup>/Pi-cotransporters the abundance of which is under hormonal control. Extracellular Pi itself also regulates the expression of cotransporters and the concentration of Pi-regulating hormones, though the signaling pathways are largely unknown. Here, we explored the mechanisms that allow renal proximal cells to adapt to changes in the concentration of Pi. **Methods:** opossum kidney (OK) cells, a model of proximal epithelia, were incubated with different concentrations of Pi in the absence/presence of phosphonoformic acid (PFA), a Pi-analogue and SLC34-inhibitor, and of inhibitors of kinases involved in hormonal control of Pi-homeostasis; cells cultured in normal media were treated with uncouplers of oxidative phosphorylation. Then, the intracellular concentration of ATP and/or the Pi-transport capacity of the cultures were analyzed. **Results:** luminal Pi regulates the Pi-transport and the intracellular ATP levels. Changes in ATP seem secondary to alterations in Pi-transport, rather than ATP acting as a signal. Adaptation of Pi-transport to high Pi was not mimicked by PFA. Transport adaptation was blocked by PFA but not by kinase inhibitors. **Conclusions:** in OK cells, adaptation of Pi-transport to luminal Pi does not depend on the same signaling pathways involved in hormonal regulation.

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## Introduction

Renal reabsorption of inorganic phosphate (Pi) is the main mechanism by which mammals keep their plasma levels of Pi constant [for review see [1, 2]]. This process takes place to a major extent along the proximal tubule and it can be regulated according to the body's requirements [for review see [3-5]]. Members of two families of Na<sup>+</sup>/Pi cotransporters,

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the SLC34 (SLC34A1/NaPi-IIa and SLC34A3/NaPi-IIc) and SLC20 (SLC20A2/Pit2), are expressed at the brush border membrane (BBM) of proximal tubular cells and mediate the secondary-active transport of Pi from the tubular lumen into the epithelial tubular cell [2, 6-10]. Their relevance is underlined by the Pi-wasting phenotype of patients with loss of function mutations in SLC34A1 or SLC34A3 [11-13]. The abundance of these transporters is controlled by the coordinated action of several humoral factors including parathyroid hormone (PTH), fibroblast growth factor 23 (FGF-23) and 1,25-dihydroxy-vitamin D<sub>3</sub> (for review see [3-5]).

In addition to hormonal alterations, renal reabsorption of Pi also adapts to acute (hours) and chronic (days) changes on dietary Pi, such that reabsorption is increased upon dietary Pi restriction and is reduced upon dietary Pi overload [9, 14-20]. Acute adaptation in response to a high Pi diet involves the internalization and lysosomal degradation of NaPi-IIa; on the other hand, relocation of NaPi-IIa from intracellular stores into the BBM seems to mediate the acute response to a low Pi diet [14, 15, 21]. These fast changes in NaPi-IIa abundance do not depend on gene regulation, whereas changes on mRNA expression have been reported upon chronic challenges [15, 19]. The expression of NaPi-IIc and Pit2 also adapts to dietary Pi but their responses are slower than those described for NaPi-IIa [9, 18]. Oral Pi also influences the plasma levels of PTH, FGF-23 and vitamin D<sub>3</sub> [22-24]. PTH acutely adapts to switches of dietary Pi as well as to intragastric administration of Pi, whereas FGF-23 and specially vitamin D<sub>3</sub> only respond upon prolonged challenges [14, 25].

Previous reports indicated that OK cells, an immortalized cell line derived from Opossum kidney, have the ability to respond to alterations in the content of Pi in the luminal medium with changes in Na/Pi-cotransport activity and NaPi-IIa abundance [26-29]. These "autonomous" changes do not represent an artifact of the immortalized model, as it was later reported that primary cultures from mouse proximal tubular cells also respond to the concentration of Pi in the culture medium [30]. Instead, they indicate that the renal epithelial cells are able to sense extracellular Pi concentrations in the absence of hormonal signals. Data obtained with the OK model showed that the response was specific for luminal Pi and required an intact microtubular network [26, 28].

The aim of this study was to gain further insights into the mechanisms that allow renal proximal cells to adapt to changes in the luminal concentration of Pi, using OK cells as model. In particular, the involvement of several kinases was investigated by using kinase inhibitors as well as by performing phosphokinase antibody arrays, whereas the role of intracellular ATP was studied by measuring changes in ATP concentration in response to luminal Pi as well as changes in Pi-transport in response to uncouplers of oxidative phosphorylation.

## Materials and Methods

### *Cell culture*

Opossum kidney (OK) cells (original 3B/2 clone or cells stably transfected with V5-tagged NaPi-IIa [31]) were maintained in a 1:1 mixture of Dulbecco's modified Eagles medium (DMEM) and Ham's F-12 (Invitrogen) supplemented with L-glutamine (2 mM), penicillin/streptomycin (50 IU/ml), NaHCO<sub>3</sub> (22 mM), HEPES (20 mM) and fetal calf serum (FCS; 10%). Cells were incubated at 37°C in a 5% CO<sub>2</sub> and 95% air atmosphere. The medium was changed every third day and the cells were split 1:3 once per week by mild trypsinization.

### *Changes in the luminal concentration of Pi*

Cells were seeded into 12 well (for uptake experiments) or 6 well (for Western blot, intracellular ATP determination and phosphokinase array analysis) plastic plates (Costar), and cultured until reaching confluency, as estimated by the appearance of domes. The day before the experiment, cells were incubated in a FSC-free medium containing either 1 mM (high Pi) or 0.1 mM Pi (low Pi) (Invitrogen). Next morning, the medium was replaced by fresh one containing either the same concentration of Pi or the corresponding switch, and the cultures were further incubated during the time indicated for each experiment.

*Treatments with phosphonoformic acid (PFA), protein kinase inhibitors and uncouplers of oxidative phosphorylation*

All treatments were performed in cells seeded into 12 well plates that were grown until reaching confluency. After overnight FCS starvation in low Pi medium, cultures were incubated during 4 hours in either low or high Pi medium without/with several concentrations of PFA (1, 2, 5 and 10 mM). PFA was purchased from Sigma and was dissolved in water.

For the kinase inhibitor studies, cells cultured overnight in FCS-free medium containing either low or high Pi were incubated in the morning without/with single dosages of inhibitors of the following protein kinases: PKA (20  $\mu$ M H-89 Dihydrochloride from Calbiochem and 2  $\mu$ M KT 5720 from Santa Cruz Biotech), PKB (10  $\mu$ M GSK690693 from Selleck and 10  $\mu$ M Akt Inhibitor XI from Santa Cruz Biotech), PKC (20  $\mu$ M Bisindolylmaleimide from Calbiochem and 5  $\mu$ M Ro 31-8220 from Santa Cruz Biotech) and ERK (30  $\mu$ M ERK inhibitor II from Santa Cruz Biotech and 25  $\mu$ M U-0126 from Enzo). After 1 hour incubation, media was replaced by fresh one containing the same concentration of inhibitors; at this point, half of the cells cultured in low Pi were switched to high Pi. Cells were incubated for 4 additional hours. Kinase inhibitors were dissolved in water or DMSO as indicated by manufacturers and the corresponding vehicle was added to the cells incubated in the absence of inhibitors.

For uncoupling of oxidative phosphorylation, cells were incubated for the indicated time periods with either 10  $\mu$ M or 2  $\mu$ M carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) or with 1  $\mu$ M valinomycin. Both chemicals, purchased from Sigma, were dissolved in absolute ethanol, and ethanol was added to the negative controls.

*Pi uptake assays*

Na<sup>+</sup>-dependent Pi uptake was determined by measuring the incorporation of radiolabeled Pi (<sup>32</sup>P, Perkin-Elmer) into OK cell monolayers in the presence/absence of Na<sup>+</sup>. For that, cells seeded into 12 well plates and subjected to the incubation protocols described above, were first washed twice with a pre-warmed uptake solution (137 mM NaCl, replaced by choline chloride in the Na<sup>+</sup>-free solution, 5.4 mM KCl, 2.8 mM CaCl<sub>2</sub>, 1.2 mM MgSO<sub>4</sub> and 10 mM HEPES/Tris, pH 7.4). Uptakes were then initiated by addition of uptake solution supplemented with 0.1 mM K<sub>2</sub>HPO<sub>4</sub>/KH<sub>2</sub>PO<sub>4</sub>, pH 7.4, as cold substrate and <sup>32</sup>Pi as a tracer. After incubation for 10 minutes at room temperature, reactions were terminated by aspiration of the uptake-media followed by several washes with ice-cold stop solution (137 mM NaCl and 14 mM Tris/HCl, pH 7.4). The cell monolayers were then solubilized in 1 ml lysis solution (0.1 M NaOH and 0.05% sodium deoxycholate). After 90 minutes incubation at room temperature on a shaker, aliquots of lysates were pipetted into scintillation tubes and mixed with scintillation cocktail (Perkin-Elmer) prior to measuring the radioactivity in a  $\beta$  counter (Packard). Each assay was performed in triplicate. Uptakes (pmol Pi/well/minute) were normalized as indicated for each experiment.

*Western blots*

OK cells (original 3B/2 clone or cells stably transfected with V5-tagged NaPi-IIa) were grown to confluence in 6 well plates. Upon washing with Ca<sup>++</sup>/Mg<sup>++</sup>-free phosphate buffered saline, cells were lysed by scraping with TBS (50 mM Tris/HCl and 120 mM NaCl, pH 8) containing Igepal (5  $\mu$ l/ml). Lysates were transferred to microcentrifuge tubes and homogenized by passing them through a 25G needle. Upon centrifugation at 4,500 rpm for 15 minutes at 4°C, supernatants were further centrifuged at 40,000 rpm for 30 minutes at 4°C. The pellets, containing total membranes, were resuspended in 50 mM mannitol and 10 mM HEPES/Tris, pH 7.2. After protein determination (Bio-Rad), 20-50  $\mu$ g of total membranes or lysates were separated on 8 or 10% SDS-PAGE, and transferred to polyvinylidene difluoride membranes (PVDF-Millipore). To inhibit nonspecific binding, membranes were first incubated at room temperature for 40 minutes in TBS (Tris 250 mM, NaCl 1.5M and 0.1% Triton) containing 5% powder milk. The PVDF membranes were then incubated overnight at 4°C in TBS/milk containing primary antibodies. After being washed, membranes were exposed during two hours at room temperature to HRP-labeled secondary antibodies diluted in TBS/milk and finally to chemiluminescent substrate (Millipore). Protein signals were detected on a LAS-4000 luminescent image analyzer (Fujifilm) and quantified by analysis software (Aida image).

#### *Antibodies*

Antibodies against the V5 epitope (1: 3,000; Invitrogen), total PKB (1: 5,000; R&D), PKB-pThr-308 (1: 5,000; R&D), PKB-pSer-473 (1: 5,000; R&D), GSK-3 $\alpha$ / $\beta$  (1: 10,000; R&D), TOR (1: 10,000; R&D), CREB (1: 10,000; R&D), HcK (1: 10,000; R&D),  $\beta$ -actin (1: 10,000; Sigma) and anti-mouse/rabbit HRP-conjugated secondary antibodies (1: 10,000; GE Healthcare) were used for Western blot at the indicated dilutions.

#### *Quantification of the intracellular concentration of ATP*

Confluent OK cells cultured in 6 well plates were incubated overnight in FCS-free media containing either high or low Pi. Next day, cells were cultured in fresh media containing the appropriate Pi switch. At several time points (30 minutes-16 hours), cultures were trypsinized with 0.1% Trypsin. After cell counting (Beckman Coulter), the intracellular concentration of ATP was measured in aliquots of 50,000 cells using the CellTiter-Glo® luminescent Cell Viability Assay kit (Promega) following the manufactures instructions. The CellTiter-Glo® kit contains a thermostable luciferase (Ultra-Glo™ Recombinant Luciferase) that in the presence of ATP, Mg<sup>++</sup> and O<sub>2</sub> oxygenates luciferin and generates a stable luminescent signal that is proportional to the amount of ATP present in the sample. The luminescent signal was measured in a luminometer (Anthos lucy3). The concentration of ATP in cell lysates was extrapolated from a standard curve obtained with samples of known ATP concentrations provided with the kit.

#### *Phosphokinase antibody array*

Confluent OK cells cultured in 6 well plates were incubated overnight with low Pi medium and then exposed next morning for 20 minutes to either low or high Pi. The kinase phosphorylation status of both cultures was compared using a Proteome Profiler Human Phospho-Kinase Array kit (R&D Systems). This kit contains nitrocellulose membranes coated with antibodies against 46 human phosphorylated kinases, with reported cross-reactivity for rat and mouse ranging from 60 to 80%, as well as all buffers and antibodies required for further manipulations. After 20 minutes incubation with either low or high Pi, cells were lysed and further processed according to the manufacturer's instructions. Upon centrifugation of lysates at 14,000g for 5 minutes at 4°C, the protein concentration in the resulting supernatants (containing cellular extracts) was determined. Aliquots of extracts containing 500 mg of proteins were added to the nitrocellulose membranes that had been previously blocked to inhibit unspecific binding. After overnight incubation at 4°C on a rocking platform, membranes were washed to remove unbound proteins, and were further incubated with a mixture of biotinylated detection antibodies and streptavidin-HRP antibodies. Chemiluminescent detection reagents were finally applied to detect spot densities. Arrays incubated with lysates from cells cultured in low and high Pi were processed in parallel. Chemiluminescent signals were quantified with the Aida image analyzer, and the density of duplicate spots representing individual phosphorylated kinases was determined. The list of target capture antibodies and their positions on the arrays can be found at <http://www.rndsystems.com/pdf/ARY003.pdf>

#### *Statistical analysis*

Statistical comparisons were done by ANOVA-Bonferroni or unpaired t- test, as indicated. P < 0.05 was considered significant. Results are presented as mean  $\pm$  SEM.

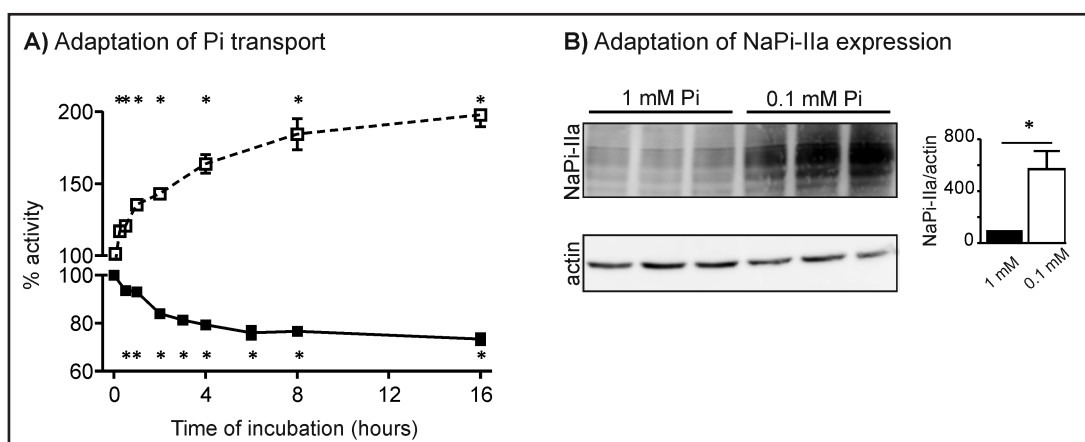
## **Results and Discussion**

### *Effect of luminal Pi in Pi-transport in OK cells*

OK cells are a renal proximal epithelial model frequently used in the field of Pi homeostasis. Similar to the proximal tubule epithelial cells, OK cells express NaPi-IIa and respond to alterations in the luminal concentration of Pi or addition of PTH with changes in Na<sup>+</sup>/Pi cotransport activity and NaPi-IIa abundance [26-29, 32].

Here, we first confirmed the time course of the adaptation of our OK cell clone in response to changes in luminal Pi (Figure 1). For that, confluent OK cultures were incubated overnight in FCS-free media containing either high (1.0 mM) or low (0.1 mM) Pi. FCS-starvation was performed in order to prevent potential activation of intracellular signaling





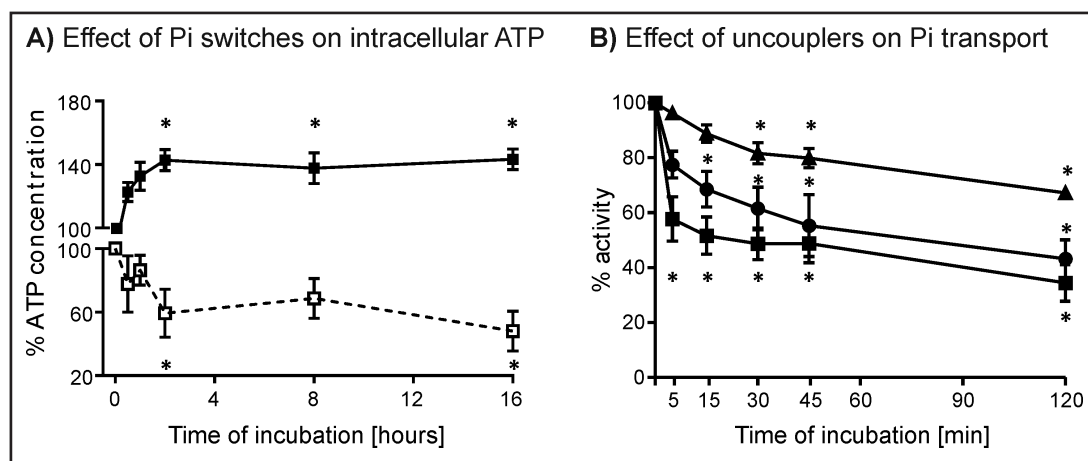
**Fig. 1.** Effect of luminal Pi in Na<sup>+</sup>-dependent Pi-transport and NaPi-IIa expression in OK cells. A) Adaptation of Pi transport: OK cells (original 3B/2 clone) were cultured overnight in media containing either 1 mM Pi (white squares) or 0.1 mM Pi (black squares). Next day, cells were incubated for the indicated times in media containing the corresponding switch on Pi concentration, and the incorporation of <sup>32</sup>P was determined. Data are expressed as percentage of values obtained immediately after switching media (120 ± 12 and 230 ± 10 pmol/well/min, for the high to low and low to high switches, respectively). Symbols represent mean ± SEM (n = 3). Statistical analysis was done by ANOVA-Bonferroni; \*P < 0.05. B) Adaptation of NaPi-IIa expression: OK cells expressing V5-tagged NaPi-IIa were incubated overnight with 1 mM Pi or 0.1 mM Pi and the amount of NaPi-IIa was quantified by Western blot using a monoclonal anti-V5 antibody. Representative Western blot and quantification analysis. The abundance of NaPi-IIa was normalized to the expression of actin. Data are expressed as percentage of values obtained in 1 mM Pi media (mean ± SEM, n = 3). Statistical analysis was done by t-test; \*P < 0.05.

cascades induced by the presence of factors in the serum. Next day, cells were cultured in fresh media containing the corresponding Pi switch and at several time intervals (15 minutes to 16 hours) the Na<sup>+</sup>-dependent incorporation of <sup>32</sup>P was measured. As shown in Figure 1A, switching from high to low Pi resulted in a fast increase in Na<sup>+</sup>-dependent Pi uptake, whereas there was a reduction in uptake when the medium was switched from low to high Pi. In both cases, 50% changes were already observed 3-4 hours after switching, slowing down later on. After 16 hours, uptakes had nearly doubled or had been reduced by 30%, respectively.

To demonstrate that changes in transport activity were paralleled by expression levels of NaPi-IIa, OK cells stably expressing V5-tagged NaPi-IIa were cultured overnight in medium containing either low or high Pi and the abundance of the cotransporter was determined by Western blot using a monoclonal V5 antibody. As shown in Figure 1B, higher V5-NaPi-IIa expression was detected in cells incubated with low Pi compared to high Pi. This observation, together with the uptake data reported above, confirms that our cells have retained the molecular machinery required to properly sense and adapt to changes in luminal Pi and that adaptation to luminal Pi is an intrinsic cell-autonomous feature of OK cells.

#### *Effect of luminal Pi in the intracellular concentration of ATP in OK cells*

Similar to renal BBMV, apical uptake of Pi into OK cells is almost exclusively mediated by Na<sup>+</sup>-dependent mechanisms, since little or no Pi incorporation is detected when Na<sup>+</sup> is replaced by choline in the uptake solution. Typical uptakes in renal BBMV are in the range of 1-5 vs 0.05-0.10 pmol Pi/μg protein/min for the Na<sup>+</sup>-dependent and independent components, respectively, whereas the values for the corresponding uptakes in OK cells obtained in this study range between 1000 and 10 pmol Pi/well/min. Therefore, luminal transport of Pi is likely fully dependent on the transmembrane gradient of Na<sup>+</sup> generated by the Na<sup>+</sup>/K<sup>+</sup> ATPase and thus may be affected by alterations in the intracellular concentration

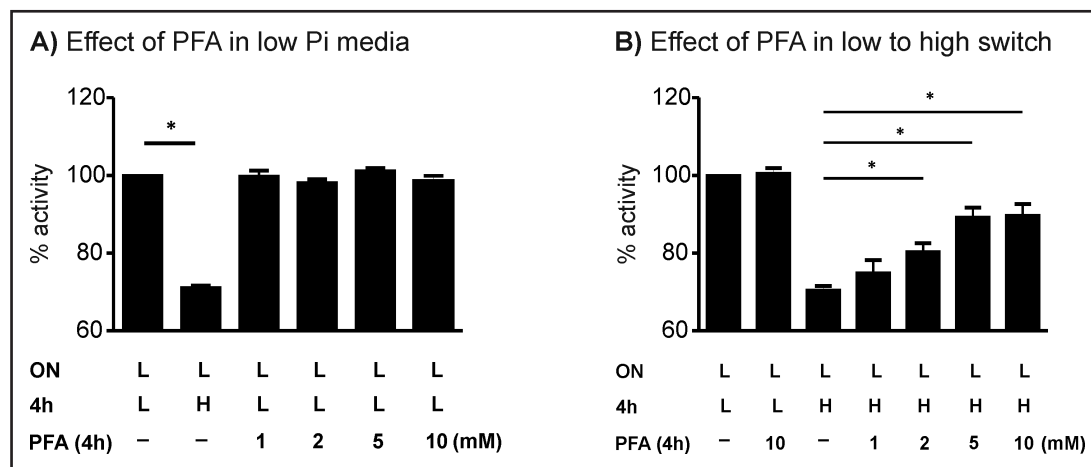


**Fig. 2.** Relationship between luminal Pi and the intracellular concentration of ATP in OK cells. A) Cells were incubated overnight in either 1 mM Pi (white squares) or 0.1 mM Pi (black squares) and then switched of media. At the indicated times, the intracellular concentration of ATP was measured with the CellTiter-Glo Luminescent assay kit. Data are expressed as percentage of values obtained immediately after switching media ( $21 \pm 4$  and  $185 \pm 25$  pmol/50000 cells, for the low to high and high to low switches, respectively). Symbols represent mean  $\pm$  SEM ( $n = 3$ ). Statistical analysis was done by ANOVA-Bonferroni; \* $P < 0.05$ . B) Cells incubated in normal media were treated with 10  $\mu$ M FCCP (circles), 1  $\mu$ M FCCP (triangles) or 1  $\mu$ M valinomycin (squares) for the indicated time before measuring the incorporation of  $^{32}$ P. Data are presented as percentage of values obtained in cells incubated with vehicle. Symbols represent mean  $\pm$  SEM;  $n = 3$  for 10  $\mu$ M FCCP and 1  $\mu$ M valinomycin and 2 for 1  $\mu$ M FCCP. Statistical analysis was done by ANOVA-Bonferroni; \* $P < 0.05$ .

of ATP. On the other hand, it has been reported that changes in Pi transport may alter the intracellular concentration of ATP [33]. Thus, reduced Pi uptake into chondrocytes of *Hyp* mice, caused by downregulation of Pit1, correlates with ATP depletion [33].

Based on the above considerations, we next investigated whether there is also a correlation in OK cells between the changes in Pi uptake induced by the luminal concentration of Pi described above and the intracellular content of ATP. For that, a similar Pi-switching protocol as the one described for the uptakes experiments was applied. At the indicated time points, cells were lysed and the concentration of ATP in the lysates was quantified. As shown in Figure 2A, the intracellular concentration of ATP increased during the first 2 hours after switching from low to high Pi, and it remained constant thereafter. In contrast, a reduction in ATP concentration was observed when medium was switched from high to low Pi. Thus, changes in the intracellular content of ATP in OK cells inversely correlates with those of Pi-transport. The time course of changes in both parameters suggests that the transport of Pi affects the ATP concentration rather than the intracellular content of ATP acting as a signal to modulate the uptake of Pi in response to changes in luminal Pi.

We further examined the above relationship by quantifying the uptake of Pi in cells incubated in normal media and treated with either FCCP or valinomycin, two uncouplers of oxidative phosphorylation. Both compounds have been shown to reduce the intracellular levels of ATP in OK cells when used at concentrations of 10  $\mu$ M and 1  $\mu$ M, respectively [34]. We found that incubation of OK cells with 10  $\mu$ M FCCP or 1  $\mu$ M valinomycin resulted in a progressive inhibition of the Pi-transport (Figure 2B). Inhibition was already detected at the shortest analyzed time point (5 minutes) and it was also observed with 2  $\mu$ M FCCP. In all cases, domes were still observed by the time uptake experiments were initiated, indicating that incubation with uncouplers had not disturbed the monolayer's tightness. The reduction in Pi-transport promoted by FCCP and valinomycin can be explained based on the expected



**Fig. 3.** Effect of PFA in the adaptation of  $\text{Na}^+/\text{Pi}$ -transport in OK cells to luminal Pi. A) Effect of PFA in low Pi media: cells were incubated overnight with 0.1 mM Pi and then treated for 4 hours with the indicated concentrations of PFA (1,2,5 and 10 mM); as control, a group was switched to 1 mM Pi for 4 hours. B) Effect of PFA in low to high Pi switch: cells were incubated overnight in 0.1 mM Pi and then exposed for 4 hours to 1 mM Pi with or without the indicated PFA concentrations. For both panels, uptakes of  $^{32}\text{P}$  were measured 4 hours after incubation with PFA (or upon media switches). Data are expressed as percentage of uptakes measured in cells incubated constantly in 0.1 mM Pi without PFA (mean  $\pm$  SEM,  $n=3$ ). Statistical analysis was done by ANOVA-Bonferroni; \* $P < 0.05$ .

dissipation of the  $\text{Na}^+$  gradient resulting from the inhibition of the  $\text{Na}^+/\text{K}^+$ -ATPase caused by ATP depletion. Indeed, reduced  $\text{Na}^+/\text{K}^+$ -ATPase activity has been reported in OK cells treated with both uncouplers [34]. In contrast, our data does not support a role of ATP as a signal for the adaptation of Pi transport to luminal Pi since, in that hypothetical case, a reduction in ATP concentration would have to mimic the effect of switching from high to low Pi and therefore would be expected to result in increased Pi transport (compare Figure 1A and 2A).

#### *Effect of PFA in the adaptation of Pi-transport in OK cells to luminal Pi*

Phosphonomformic acid (PFA) is a Pi analog that acts as a competitive inhibitor of SLC34 NaPi cotransporters in intestine and kidney [35, 36]. Previous experiments showed that administration of PFA in vivo induces phosphaturia [37]. However, the  $\text{Na}^+/\text{Pi}$  transport as well as the binding of radiolabeled PFA was similar in BBMVs isolated from PFA-treated and control rats, suggesting that phosphaturia was caused by direct inhibition of the (at the time unknown) renal  $\text{Na}^+/\text{Pi}$  cotransporter. The same group also reported that addition of PFA in the uptake solution inhibits the  $\text{Na}^+/\text{Pi}$  cotransport into renal BBMVs in a dose-dependent, reversible and specific manner [36]. These latter observations were also replicated in OK cells [29, 38]. Experiments in *Xenopus laevis* oocytes injected with NaPi-IIa cRNA indicated that in the presence of PFA two  $\text{Na}^+$  ions can still bind to the cotransporter, but subsequent binding of Pi is blocked [39].

Since PFA behaves as a Pi analogue, here we first analyzed whether addition of PFA to the culture medium can mimic the effect of high Pi described above, i.e. leads to reduction of  $\text{Na}^+$ -dependent uptake of Pi. For that, OK cells were incubated overnight with low Pi and then treated for 4 hours with increasing concentrations of PFA added to fresh low Pi medium. As positive control for adaptation, a group of cells was switched for 4 hours to high Pi (in absence of PFA). As shown in Figure 3A, addition of PFA to the low Pi media failed to induce changes in the  $\text{Na}^+$ -dependent uptake of Pi, even at high concentrations, whereas the expected reduction was observed in cells switched to high Pi. Thus, PFA alone does not trigger a Pi-responsive adaptation.



Next, we analyzed whether PFA interferes with the reduction of Pi uptake induced by high Pi. For that, cells incubated overnight with low Pi were switched for 4 hours to high Pi in the absence/presence of increasing PFA concentrations. As negative control, a group of cells was maintained in low Pi and incubated with 10 mM PFA. As shown in Figure 3B, the expected decrease in Na<sup>+</sup>-dependent Pi uptake was observed in the cells switched from low to high Pi in the absence of PFA. This decrease was blocked by PFA in a dose dependent manner, with significant blocking achieved with PFA concentrations ranging from 2 to 10 mM.

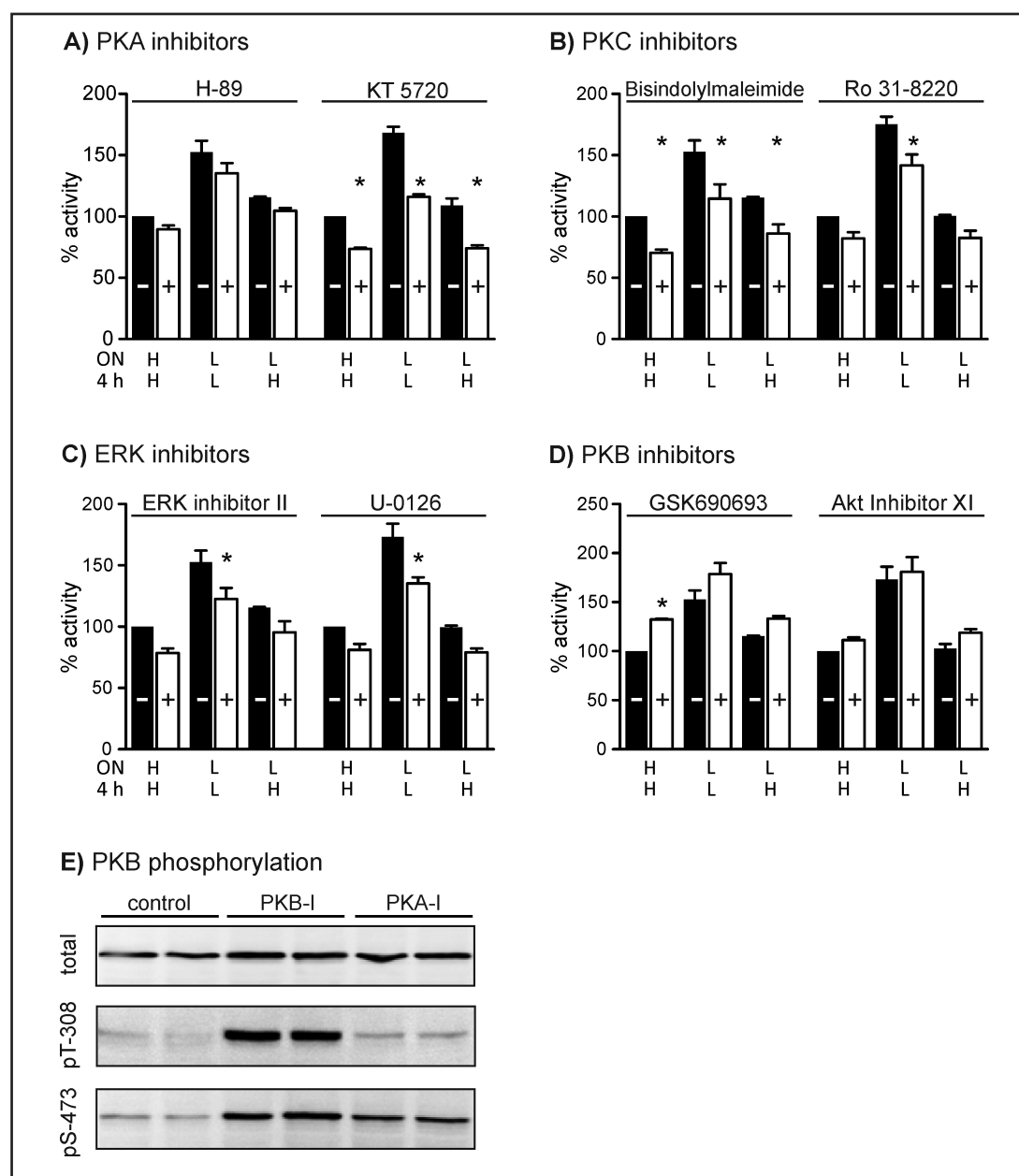
The effect of PFA on the reduction of Pi transport induced by high Pi may have several potential explanations. On one hand, by inhibiting Na<sup>+</sup>/Pi-cotransporters, PFA may reduce the amount of Pi loaded into the cell compared with the levels reached in its absence; this possibility would suggest that changes in intracellular Pi are required to activate sensing mechanism(s). Alternatively, PFA may directly interfere with components of the cell membrane involved in Pi-sensing.

#### *Effect of selected kinases inhibitors in the adaptation of Pi-transport in OK cells to high luminal Pi*

Studies addressing the intracellular mechanisms that mediate regulatory changes of renal Na<sup>+</sup>/Pi cotransport (and of Na<sup>+</sup>/Pi-cotransporters) have mainly focused in the effects triggered by PTH. Binding of PTH to receptors in proximal tubular cells activates several kinases including PKA, PKC and ERK leading to endocytosis and lysosomal degradation of NaPi-IIa [40, 41]. In addition, a role of PKB in hormonal regulation of transport of Pi has also been proposed [42]. The implication of these kinases in the OK cell model was demonstrated in early reports [43-47].

Since endocytosis and lysosomal degradation of NaPi-IIa is also observed in response to high dietary Pi [21], here, we first analysed whether the adaptation of OK cells to high luminal Pi is sensitive to inhibition of the kinases mentioned above. To this end, the effect of inhibitors of PKA, PKC, ERK and PKB on Pi-transport was analyzed in cells constantly exposed to either low or high Pi media as well as in cells switched from low to high Pi for 4 hours. Inhibitors were used at concentrations either previously shown to block the corresponding kinases in OK cells [43-47] or about two orders of magnitude higher than the Ki provided by the manufacturers. Doses were still observed by the time uptake experiments were initiated. PKA was inhibited by addition of H-89 Dihydrochloride or KT5720 to the culture media. Although the reductions on Pi-transport induced by H-89 did not reach statistical significance, incubation with KT 5720 resulted in significantly reduced incorporation of Pi in all the media analyzed (Figure 4A). Similarly, the effects of two different PKC inhibitors, namely Bisindolylmaleimide and Ro 31-8220, were tested. As shown in Figure 4B, Bisindolylmaleimide led to significant inhibition of the Pi-transport activity regardless of the luminal concentration of Pi, whereas the effect of Ro 31-8220 was significant only in cells incubated in low Pi. Blocking ERK with either the ERK inhibitor II or the U-0126 compound resulted in reduced Pi-transport, though in both cases only the changes detected in low luminal Pi reached statistical significance (Figure 4C). In contrast to the above inhibitors, inhibition of PKB with GSK690693 resulted in significant higher uptakes in cells cultured in high Pi (Figure 4D); however, addition of a second PKB inhibitor (Akt Inhibitor XI) failed to induced significant changes in Pi incorporation in any media (Figure 4D). A recent study showed that the GSK690693 inhibitor blocks PKB signaling by inducing the phosphorylation of Thr308 and Ser473 [48]. We also observed phosphorylation of both residues in GSK690693-treated OK cells (Figure 4E), confirming the action of the inhibitor in our cell model, whereas the state of phosphorylation of both residues was not affected upon addition of the Akt Inhibitor XI (data not shown).

Together, these observations suggest that some kinases (especially PKA and PKC) may regulate in a positive fashion the constitutive transport of Pi in OK cells, regardless of the concentration of luminal Pi. However, our data does not support a role of any of the four



**Fig. 4.** Effect of selected kinases inhibitors in the adaptation of Pi-transport in OK cells to high luminal Pi. A-D) Cells were incubated overnight with either 1 mM Pi (H) or 0.1 mM Pi (L). In the next morning, cultures were pre-incubated for 1 hour in the absence (black bars) or presence (white bars) of inhibitors of the following kinases: A) PKA: H-89 Dihydrochloride and KT 5720, B) PKC: Bisindolylmaleimide and Ro 31-8220, C) ERK: ERK inhibitor II and U-0126 and D) PKB: GSK690693 and Akt Inhibitor XI. After 1 hour, media was aspirated and replaced by fresh one containing the same vehicle/kinase inhibitor; at this point, cells cultured in 0.1 mM Pi were either switched to 1 mM Pi-containing media or kept in the presence of 0.1 mM Pi. Uptakes of  $^{32}\text{P}$  were measured after 4 hours. Data are expressed as percentage of the incorporation measured in cells constantly incubated in 1 mM Pi (mean  $\pm$  SEM,  $n=3$ ). Statistical analysis was done by ANOVA-Bonferroni; \* $P < 0.05$  between cells incubated in the absence or presence of inhibitors but exposed to the same luminal Pi conditions. E) Western blots: lysates from cells cultured in 1 mM Pi and treated with/without PKB (GSK690693) and PKA (H-89 Dihydrochloride) inhibitors were incubated with antibodies against total PKB, p-Thr308 and p-Ser473.

investigated kinases in the adaptation to high luminal Pi, since the changes detected in cells switched from low to high Pi were indistinguishable from those triggered by the inhibitors in cells constantly cultured in either medium.

#### *Effect of luminal Pi in the pattern of phosphorylation of several kinases*

Because the inhibitor data discussed above failed to confirm the implication of preselected kinases in the cellular response to high luminal Pi, we next extended these studies by using a commercially available phosphokinase antibody array. This array provides information on the phosphorylation status of 46 human kinases; the manufacturers reported cross-reactivity for rat and mouse orthologues ranging from 60 to 80%. We thus assumed that Opossum phospho-kinases may be detectable.

Since the phosphorylation status of kinases changes very fast in response to stimuli, OK cells were first incubated overnight with low Pi and then exposed to either low or high Pi for just 20 minutes. Quantification of chemiluminescent signals indicated that switching to high luminal Pi resulted in significant reductions in the phosphorylation levels of 6 kinases: GSK-3 $\alpha/\beta$ , TOR, CREB, Src, Fyn and Hck (Figure 5). However, these changes could not be reproduced by Western blot using the individual anti-phospho-antibodies, as these antibodies did not specifically label proteins of the expected molecular weight in the OK cell lysates (data not shown).

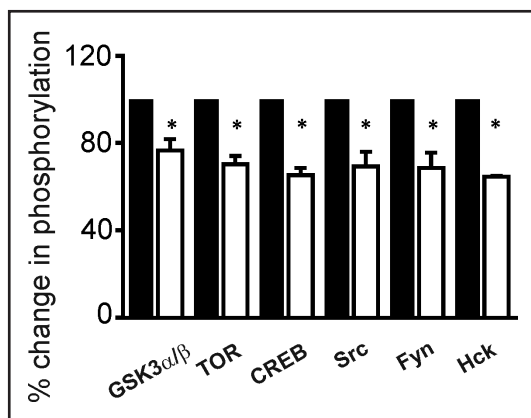
Based on this failure of the human antibodies to recognize the Opossum orthologues, the apparent regulations of the phosphorylation status of the 6 kinases reported above are probably the result of non-specific interactions between the human antibodies spotted on the nitrocellulose membranes with proteins of the OK cells lysates.

## Conclusion

In summary we have shown that: a) changes in the luminal concentration of Pi modify the transport of Pi as well as the intracellular levels of ATP in OK cells, b) changes in ATP seem to be secondary to alterations in Pi-transport, rather than the intracellular content of ATP acting as a signal, c) PFA does not mimic the adaptation of Pi-transport induced by high Pi; however, it blocks the transport response triggered by high Pi, and d) inhibitors of PKA, PKB, PKC and ERK do not impair the downregulation of Pi-transport in response to high luminal Pi. Therefore we conclude that in OK cells, adaptation of Pi-transport to changes in luminal Pi does not depend on the same signaling pathways involved in its hormonal regulation.

## Disclosure Statement

The authors state that they have no conflicts of interest.



**Fig. 5.** Effect of luminal Pi in the pattern of phosphorylation of several kinases (phosphokinase antibody array). Quantification analysis: chemiluminescent signals were normalized to the values obtained with lysates from cells kept in 0.1 mM Pi. Ratios for the 6 kinases (mean  $\pm$  SEM, n=3) which phosphorylation status was regulated by the content of Pi in the media are shown in the graph: black bars= 0.1 mM Pi, white bars= 1 mM Pi. Statistical analysis was done by t-test; \*P < 0.05.

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